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Development of Crystalline Peroxisomes in Methanol-Grown Cells of the Yeast *Hansenula polymorpha* and Its Relation to Environmental Conditions

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Abstract. The development of peroxisomes has been studied in cells of the yeast *Hansenula polymorpha* during growth on methanol in batch and chemostat cultures. During bud formation, new peroxisomes were generated by the separation of small peroxisomes from mature organelles in the mother cells. The number of peroxisomes migrating to the buds was dependent upon environmental conditions. Aging of cells was accompanied by an increase in size of the peroxisomes and a subsequent increase in their numbers per cell. Their ultimate shape and substructure as well as their number per cell was dependent upon the physiological state of the culture. The change in number and volume density of peroxisomes was related to the level of alcohol oxidase in the cells. Development of peroxisomes in cells of batch cultures was accompanied by an increase in size of the crystalline inclusions in the organelles; they had become completely crystalline when the cells were in the stationary phase. Peroxisomes in cells from methanol-limited chemostat cultures were completely crystalline, irrespective of growth rate. Results of biochemical and cytochemical experiments suggested that alcohol oxidase is a major component of the crystalline inclusions in the peroxisomes of methanol-grown *Hansenula polymorpha*. Possible mechanisms involved in the ultrastructural changes in peroxisomes during their development have been discussed.

Key words: Peroxisome – Methanol – Cytochemical staining – Yeast – *Hansenula polymorpha*.

It is now well established that adaptation of certain yeasts to growth with methanol as the only carbon and energy source is accompanied by the development of peroxisomes (van Dijken et al., 1975a; Sahm et al., 1975; Fukui et al., 1975a). Biochemical and cytochemical evidence has shown that these organelles not only contain enzymes involved in the oxidation of methanol, namely alcohol oxidase and catalase, but also amino acid oxidase and hydroxy acid oxidase (Fukui et al., 1975b; Veenhuis et al., 1976). However, polyacrylamide gel electrophoresis of peroxisomal protein from methanol-grown *Candida boidinii* suggested that, at least in this yeast, alcohol oxidase and catalase were the main components of the organelles (Roggenkamp et al., 1975).

At the ultrastructural level a striking difference appeared to exist between peroxisomes of batch- and chemostat-grown cells of the organisms. In batch cultures of different *Hansenula*, *Pichia*, *Kloeckera*, and *Candida* strains the peroxisomes in the cells appeared as rounded or irregularly shaped organelles with a crystalline nucleus (van Dijken et al., 1975a; Fukui et al., 1975a; Tanaka et al., 1976; Roggenkamp et al., 1975). On the other hand, cells of *Hansenula polymorpha*, grown in a methanol-limited chemostat, contained peroxisomes which were almost cubic of form and showed a completely crystalline substructure (van Dijken et al., 1975b; Veenhuis et al., 1976). These findings suggested that the morphology and the degree of crystallinity of the peroxisomes may depend on growth conditions. This prompted us to study the ultrastructure of peroxisomes in relation to various environmental conditions. Since morphological differences observed between peroxisomes in one cell may reflect differences in the stages of development of single organelles, this aspect was also taken into account.

This paper describes the results of an ultrastructural and biochemical analysis of the development of peroxisomes in the yeast *Hansenula polymorpha*, when grown on methanol under various conditions. Attention is focused on the formation of crystalloids.

Abbreviations. DAB = 3,3′-diaminobenzidine; OD = optical density (663 nm).

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Materials and Methods

Microorganism and Cultivation. Hansenula polymorpha de Morais et Maya CBS 4732 was used in all experiments. The organism was grown in methanol-limited chemostat cultures at 37° C on the mineral medium of van Dijken et al. (1976). The dissolved oxygen tension was kept at approximately 50% of air saturation. In studies of batch cultures the organism was grown in 500 ml Erlenmeyer flasks containing 100 ml of a medium with the following composition: KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1.5 g; trace elements according to Vishniac and Santer (1957), 0.2 ml; yeast extract, 0.5 g; methanol, 4 g and distilled water, 1 l. Methanol-grown cells which had been in the stationary phase for 5–10 h were used as the inoculum.

Measurement of the Rate of Oxidation of Excess Methanol by Whole Cells ($Q_{O_2}^{max}$). $Q_{O_2}^{max}$ of washed cell suspensions was determined with a polarographic oxygen electrode as described by van Dijken et al. (1976).

Enzyme Assays. The preparation of cell-free extracts and the estimation of alcohol oxidase activity were as described previously (van Dijken et al., 1976). The activity of alcohol oxidase was expressed as μmoles of oxygen consumed/min × mg protein. Catalase was assayed by the spectrophotometric method of Lück (1963). Catalase activity was expressed as ΔE_{240}/min × mg of protein. Protein concentrations in cell-free extracts were determined by the method of Lowry et al. (1951).

Preparation of Spheroplasts. Spheroplasts were prepared by treatment of whole cells with "Zymolyase" (Kitamura et al., 1971) for 20 min at 37° C according to the procedure of Osumi et al. (1975). For osmotic shock treatment, a suspension of spheroplasts was centrifuged at 2500 g and the pellet resuspended in 50 mM K-phosphate buffer pH 7.2 for 1 min before fixation with glutaraldehyde.

Fixation Techniques. Whole cells were harvested, washed once with distilled water and fixed with 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 for 30 min at 0° C. The osmotic strength of this fixative was adjusted with sorbitol to the same value as that of the spheroplast suspension.

Cytochemical Staining and Postfixation. The location of catalase activity in the cells was detected with 3,3′-diaminobenzidine (DAB) and hydrogen peroxide or, alternatively with DAB and methanol (Veenhuis et al., 1976). The in situ activity of the peroxisomal oxidases was visualized using the cerium technique (Veenhuis et al., 1976). Whole cells were postfixed in a solution of 1% OsO_4 and 2.5% $K_2Cr_2O_7$ in 0.1 m cacodylate buffer, pH 7.2 for 45 min. After dehydration in a graded ethanol series the material was embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

Morphometrical Analysis of Thin Sections. The average number of peroxisomes in thin sections of cells was estimated by at random counting. For each sample at least 500 cell profiles were counted. Volume densities of peroxisomes in the cytoplasm were estimated with the point counting technique according to Weibel and Bolender (1976). The final magnification of the micrographs was 7500 ×; the point spacing of the test system was 2.5 cm. Student's *t*-test was used for statistical analysis.

Results

Quantitative Aspects of Peroxisome Development in Batch Cultures

When methanol-grown cells of *Hansenula polymorpha*, which had been in the stationary phase for 10 h were inoculated in fresh medium, growth started after a lag of approximately 3 h (Fig. 1 A). In the course of these experiments, significant changes were observed in the capacity of cells to oxidize excess methanol ($Q_{O_2}^{max}$). During the lag phase the $Q_{O_2}^{max}$ rapidly declined to 30% of its original value. After growth had started, the capacity to oxidize methanol increased to reach an optimum after the culture entered the stationary phase and then declined again (Fig. 1 A). This behaviour of whole cells with respect to the oxidation of methanol is probably a reflection of the activity of peroxisomal alcohol oxidase which followed a similar pattern throughout the growth curve. The profile of catalase activity followed that of alcohol oxidase except that the level of this enzyme reached its maximal activity in the midexponential growth phase (Fig. 1 B).

In order to determine whether these striking changes in enzyme activities were accompanied by a change in number and volume density of the peroxisomes, a morphometrical analysis was carried out on thin sections of $KMnO_4$-fixed cells from different stages of growth. It was found that although the activity of alcohol oxidase and catalase decreased by 70% during the lag, the number and volume density of the peroxisomes in the cells remained unchanged during this period (Fig. 1 C). After growth had started the volume density of the organelles decreased by 70% during the first hours and then increased again. The number of peroxisomes varied accordingly; the initial decrease in volume density was accompanied by a decrease in number while parallel with the subsequent increase in volume density also a gradual increase in number was observed (Fig. 1 C). An unexpected result was the behaviour of the mitochondria during growth. The total mitochondrial volume density sharply increased during the first phase of exponential growth and then decreased again (Fig. 1 C).

The results described above do not permit conclusions to be drawn with respect to the quantitative aspects of synthesis of cell organelles in individual cells since an at random analysis was performed both on cells which were present in the inoculum and on the daughter cells generated during exponential growth. Therefore, a separate analysis was carried out on the older cells which came from the inoculum as well as on newly formed cells. This was possible because the two types of cells differed in among others the thickness of the cell wall, the electron density of the cytoplasm and the presence of bud scars. The results showed that the cells of the inoculum were not subject to significant morphological changes. In these cells the number and the volume density of the peroxisomes as well as the growth (Table 1). However, significant changes were observed in the newly formed cells (Fig. 1 D). The volume density of the peroxisomes, amounting to only

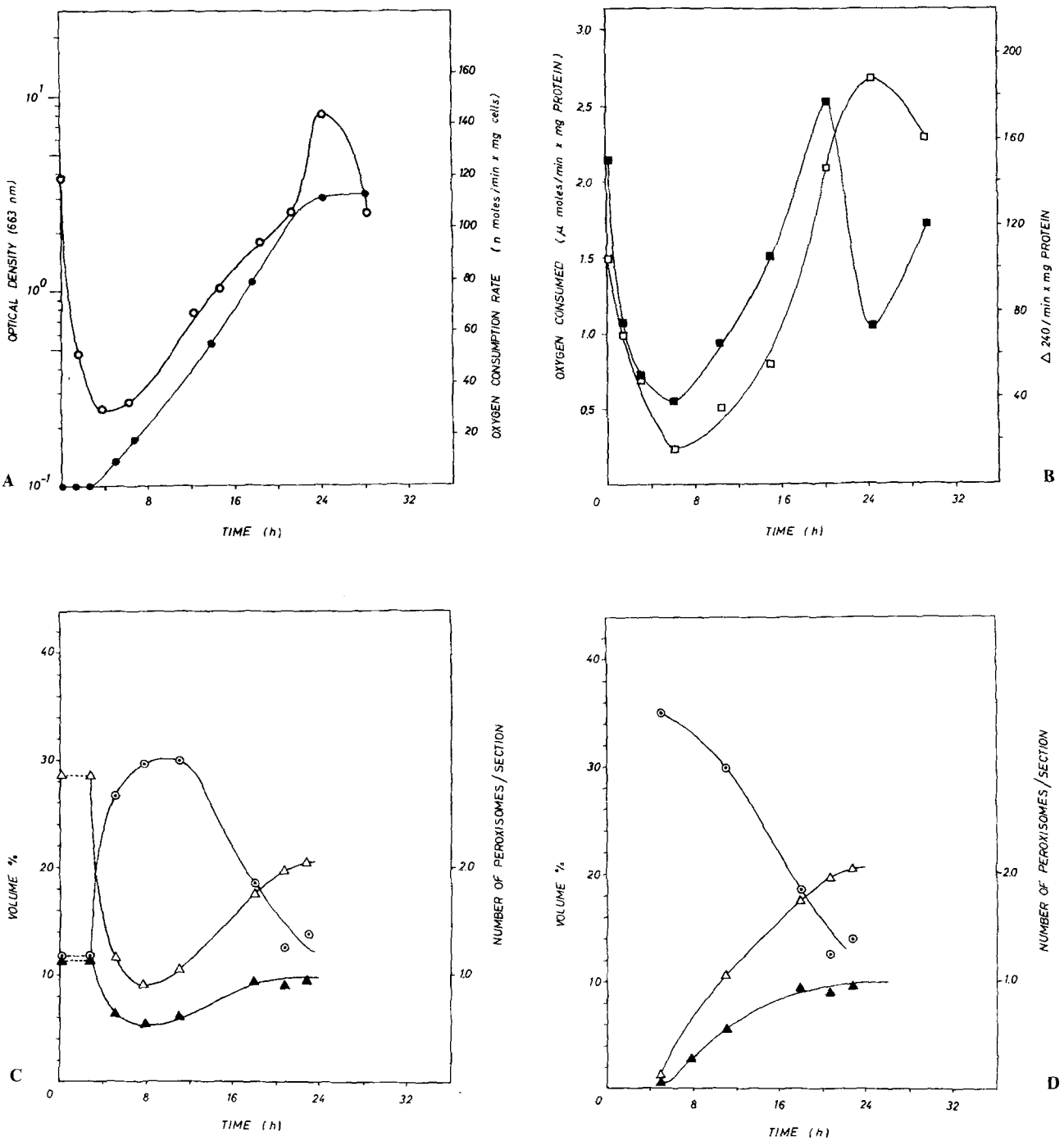
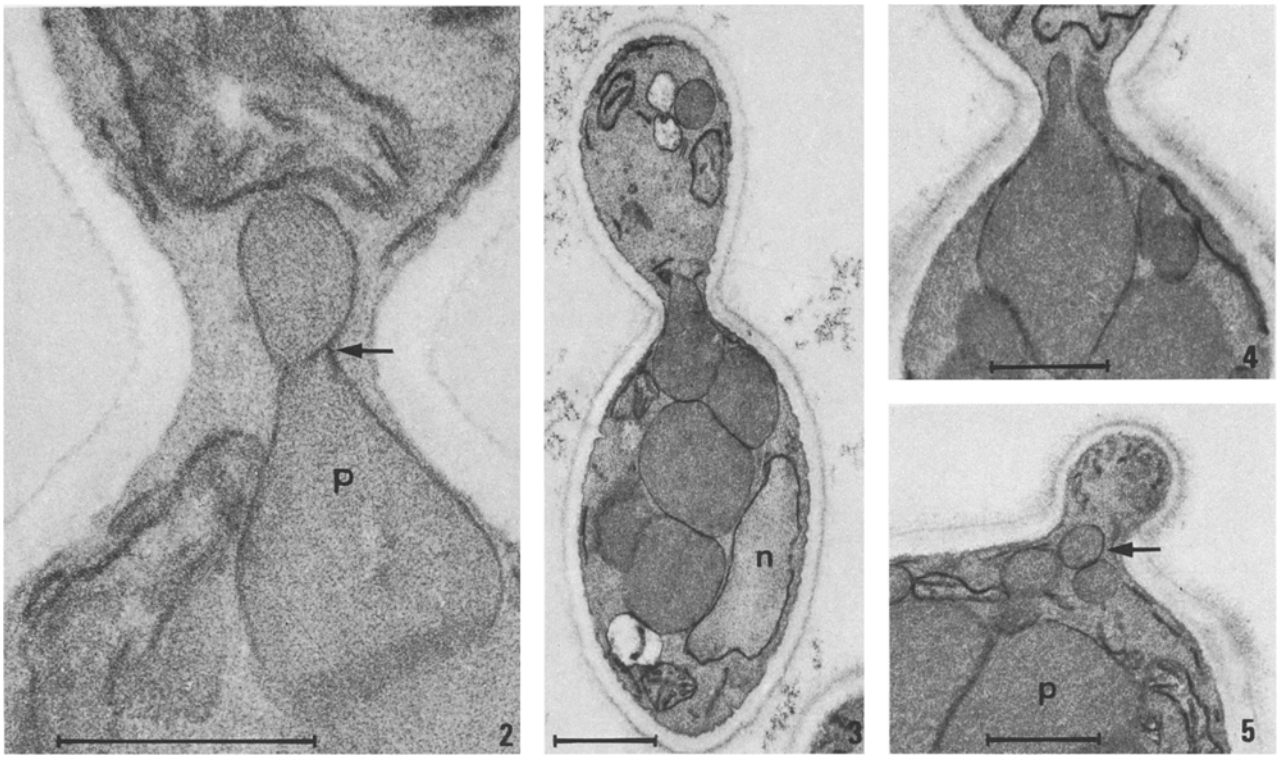


Fig. 1 A–D. Growth, enzyme profiles and organelle synthesis in batch cultures of *Hansenula polymorpha* in the presence of methanol. Cultures were inoculated with methanol-grown cells from the stationary growth phase (see "Materials and Methods"). The volume density of peroxisomes and mitochondria is expressed as percentage of the cytoplasmic volume. **A** Growth (●) and methanol-oxidizing capacity ($Q_{O_2}^{max}$) of washed cell suspensions (○). **B** Activity of alcohol oxidase (□) and catalase (■) in cell-free extracts. **C** Volume density of peroxisomes (△) and mitochondria (⊙) and the average number of peroxisomes per section (▲). **D** Volume density of peroxisomes (△) and mitochondria (○) and the average number of peroxisomes per section (▲) in the newly formed cells only

0.15% in the early exponential growth phase (OD_{663} = 0.16), increased gradually during the growth curve and made up 20% of the cytoplasmic volume in the stationary phase cells. In contrast, the mitochondrial volume density decreased from 35% in cells at an optical density of 0.16 to 12% in stationary phase cells. The increase in peroxisomal volume density was accompanied by an increase in the number of these organelles. However, since the volume density of the peroxisomes increased 10 times as fast as their number,



Electron Micrographs. Abbreviations: *b* bud; *mc* mother cell; *m* mitochondria; *n* nucleus; *p* peroxisome. Cells were fixed or postfixed with $KMnO_4$ and taken from batch cultures unless stated otherwise. The marker represents 0.5μ

Fig. 2. Detail of a cell illustrating the division of peroxisome in the neck between mother cell and bud. Note the membrane separating the two peroxisomes (*arrow*)

Figs. 3–5. Division of peroxisomes in chemostat-grown cells (dilution rate 0.17 h^{-1}). A survey is given in Figure 3; Figure 4 shows a detail of a dividing organelle in the neck between mother cell and bud. Figure 5 illustrates the migration of small peroxisomes separated from mature organelles in the mother cell into the developing bud

it became evident from these data that the peroxisomes had increased in size during exponential growth of the culture.

Qualitative Aspects of Peroxisomes Development in Batch Cultures

The development of the peroxisomes during growth of *Hansenula polymorpha* in batch cultures and the ultrastructural changes associated with this process, were examined in $KMnO_4$-fixed cells as well as in spheroplasts, fixed with glutaraldehyde and osmiumtetroxide. It was found that the peroxisomes of cells used as the inoculum, which had been stationary for 10 h, were cubic of form and had a completely crystalline matrix. Bud formation by these cells when placed in fresh medium followed the typical pattern of that described for ascomyceteous yeasts (Kreger-van Rij and Veenhuis, 1972). Our observations suggest that the peroxisomes in the buds originate from the mature organelles in the mother cell. In general two processes were observed (Figs. 2–5). Firstly, separation of small peroxisomes from large organelles occurred in the neck between mother cell and bud (Figs. 2–4). In this case the small peroxisome was already located in the bud before it had completely separated from the large peroxisome. Secondly, separation of small peroxisomes from mature organelles in the mother cell was observed (Fig. 5), followed by their migration into the bud. The small peroxisomes generated by either process were not

Table 1. Number and volume density of peroxisomes and mitochondria in cells of the inoculum (see "Materials and Methods") during the early logarithmic growth phase

OD_{633}	Number of peroxisomes	Volume density of peroxisomes	Volume density of mitochondria
0.10	1.2	28.4	10.7
0.16	1.4	32.3	8.5
0.30	1.2	30.0	9.3

The number of peroxisomes is given as average number per section. Volume densities are expressed as percentage of the cytoplasmic volume. Statistical analysis of the data listed in this table showed no significant differences between the 3 samples

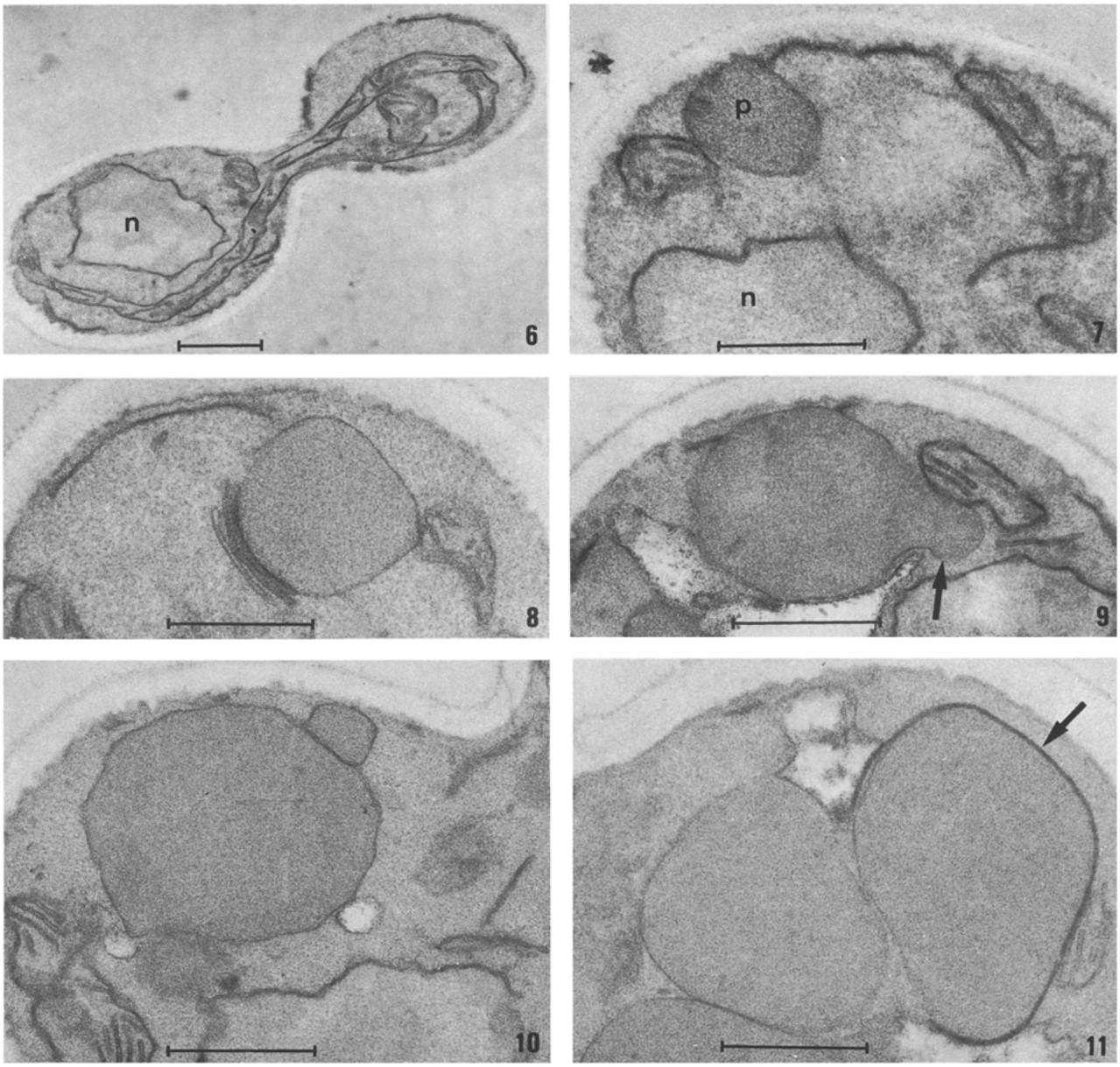
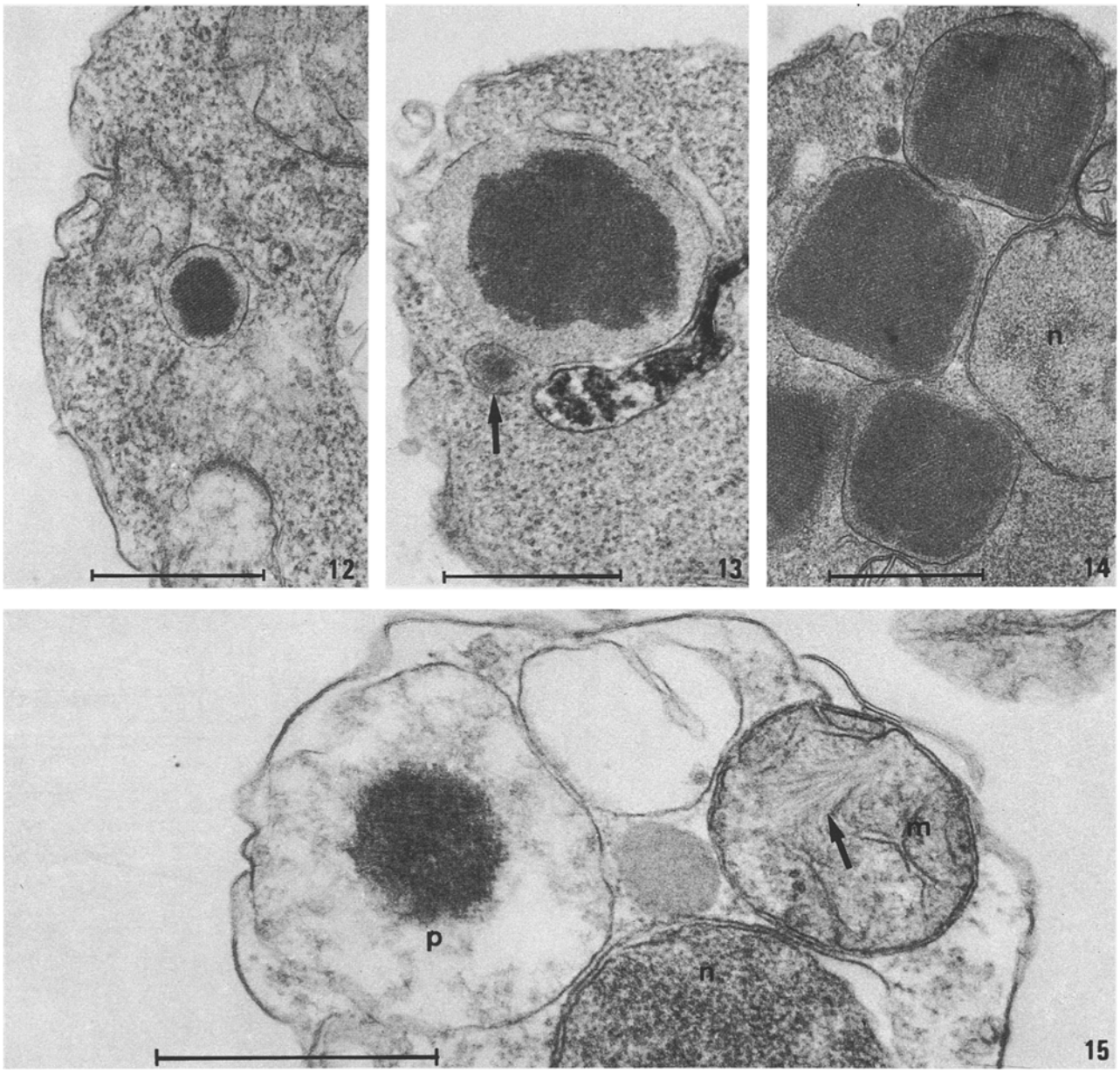


Fig. 6. A typical example of a budding cell from the early logarithmic growth phase. Note the large mitochondrion. Peroxisomes are not visible in this section

Figs. 7–11. Micrographs printed at the same magnification to illustrate growth and development of peroxisomes. Note the budding of a mature organelle (Fig. 9; *arrow*) which results in the formation of a small peroxisome which remains attached to the large organelle (Fig. 10). Association of peroxisomes with endoplasmatic reticulum and mitochondria is also visible (Figs. 7–9 and 11)

completely crystalline but showed a crystalline nucleus surrounded by a granular matrix (Fig. 12). Young cells from the first generation of growth characteristically contained huge mitochondria (Fig. 6). In general, these buds contained one or two small peroxisomes, always of round shape, which were located aside the lateral wall (Fig. 7). At this stage of the culture these peroxisomes were typically closely associated with endoplasmatic reticulum and mitochondria (Figs. 7–9). As growth progressed, the peroxisomes increased in size, while associations with endoplasmatic reticulum, mitochondria or even with Golgi-like structures were still evident (Fig. 8). Growth of peroxisomes was associated with an increase in the size of the crystalline nucleus (Fig. 13); however, completely crystalline organelles were not observed in exponentially growing cells. In young cells, the number of peroxisomes increased because small organelles separated from the more



Figs. 12–14. The ultrastructure of developing peroxisomes as observed in spheroplasts, fixed with glutaraldehyde and OsO_4 + $K_2Cr_2O_7$. In Figure 12 a small organelle is shown in a cell from the early logarithmic growth phase (compare Figs. 4 and 5). Figure 13 shows a detail of a cell from the mid exponential growth phase (compare Fig. 10). Note the presence of a crystalline inclusion in the young organelle (*arrow*). Figure 14 shows the characteristic features of peroxisomes of cells from the early exponential growth phase

Fig. 15. Detail of a spheroplast subjected to osmotic shock. The peroxisome has swollen considerably but the crystalline inclusion is apparently intact. The matrix of the non-crystalline part of the peroxisome is less electron dense than that of a corresponding intact organelle (compare Fig. 9). In the mitochondrion the DNA has probably condensed (*arrow*). Fixation: glutaraldehyde; OsO_4 + $K_2Cr_2O_7$

mature peroxisomes (Figs. 9 – 11). These small organelles remained attached to the mature peroxisome and also contained a crystalline nucleus (Fig. 13). As before, associations with endoplasmatic reticulum and mitochondria were frequently observed at this stage (Fig. 11). In the decelerated growth phase, the peroxisomes became more rectangular of form, although the matrix was not completely crystalline (Fig. 14). Completely crystalline peroxisomes were observed in cells which, similarly to the cells of the inoculum, had been in the stationary phase for several hours (Fig. 20). An additional remark with respect to the number of peroxisomes appearing in the developing buds has to be made. It was observed that this number was dependent

upon the stage of growth of the culture. In the early exponential growth phase budding was generally accompanied by migration of 1 or 2 peroxisomes into the daughter cells, whereas during later stages of growth 2–4 peroxisomes were transferred into the buds.

In situ Activity of Peroxisomal Enzymes

The in situ activity of peroxisomal enzymes during growth was assessed with cytochemical staining techniques using spheroplasts prepared from cells at different stages of growth. The observed decrease, in cell-free extracts, of alcohol oxidase and catalase activity during the lag (Fig. 1 B) was supported by cytochemical evidence, since the matrix of the peroxisomes was only partly stained or even not stained at all after appropriate incubations. The progressive inactivation of these peroxisomal enzymes during this period was not accompanied by any change in the substructure of the organelles because after glutaraldehyde/OsO_4 fixation the matrix showed a completely crystalline substructure.

In order to study the enzymic composition of the crystalline inclusions in exponential growing cells, an attempt was made to modify the composition of the organelles artificially before analyzing the result with cytochemical staining techniques. In Figure 15 part of a spheroplast of a cell from the exponential growth phase is shown which was subjected to an osmotic shock. It can be seen that the peroxisome is considerably enlarged as a result of this procedure, but the crystalline core of the organelle remained intact. The non-crystalline matrix of the peroxisomes was less electron-dense than that of intact peroxisomes, indicating that the contents of the organelle had either been diluted or that part of it had leaked into the cytoplasm. Figure 16 shows the result of the cytochemical staining for alcohol oxidase activity with $CeCl_3$ in a mixture of swollen and intact spheroplasts. The non-crystalline part of the peroxisomes in intact spheroplasts stained heavily as compared to the crystalloid (Figs. 16 and 17). In swollen spheroplasts, however, staining of the non-crystalline matrix of the peroxisome was not observed, although staining of the crystalline part of the organelle was still apparent. Apart from residual activity in the crystal, alcohol oxidase activity was also detected in the cytoplasm of swollen spheroplasts (Fig. 16). The mobility and distribution of other peroxisomal oxidases in swollen and intact spheroplasts was identical to that of alcohol oxidase (Figs. 18 and 19). Among these was urate oxidase, an enzyme not present in peroxisomes of chemostat-grown cells (Veenhuis et al., 1976). In contrast to the results obtained with the oxidase, the activity of catalase almost completely disappeared from the peroxisomes when spheroplasts were subjected to an osmotic shock (Fig. 22). This indicated that catalase activity, which is present throughout the peroxisomes, although more pronounced in the non-crystalline matrix (Fig. 22), had leaked from the matrix as well as from the crystalline inclusions.

Cytochemical staining of peroxisomal oxidases and catalase on cells of the stationary growth phase revealed that the enzymes were present throughout the peroxisomes, which were completely crystalline. The very small zone between the crystal and the peroxisomal membrane stained always more intensive (Figs. 20 and 21). A notable exception with respect to the complete staining of the peroxisomes was found in dividing peroxisomes during migration from mother cell into the bud in cells of the exponential growth phase. As with the results obtained during continuous cultures studies (Veenhuis et al., 1976), it was found that peroxisomes present in the bud did not show alcohol oxidase activity unless bud formation was complete (Fig. 23). Catalase activity, however, remained evident in peroxisomes migrating to the bud (Fig. 24).

Development of Peroxisomes in Chemostat Cultures

It has been reported (van Dijken et al., 1976) that during growth of *Hansenula polymorpha* in methanol-limited chemostat cultures, the alcohol oxidase content of the cells increased with decreasing growth rate to up to 20% of the soluble protein, whereas the level of catalase in the cells was independent of the growth rate. Similar to the situation in batch cultures, the increase in methanol oxidase activity was correlated with an increase in the volume of peroxisomes in chemostat-grown cells (Table 2). The number of peroxisomes – as well as the volume density of the mitochondria – was independent of the growth rate. Therefore, the increasing volume density of peroxisomes at low growth rates must be explained by an increase in the size of the

Table 2. Number and volume density of peroxisomes and mitochondria in cells growing in methanol-limited chemostat cultures at various dilution rates

Dilution rate (h^{-1})	Number of peroxisomes	Volume density of peroxisomes	Volume density of mitochondria
0.03	2.6	48.4	8.5
0.10	2.8	37.0	8.4
0.17	2.6	34.1	8.8

The number of peroxisomes is given as the average number per section. Volume densities are given as percentage of the cytoplasmic volume. Statistical analysis of the data presented in this table showed that only the difference in volume density of peroxisomes in cells growing at a dilution rate of $0.03\,h^{-1}$ and that of the other two dilution rates was significant

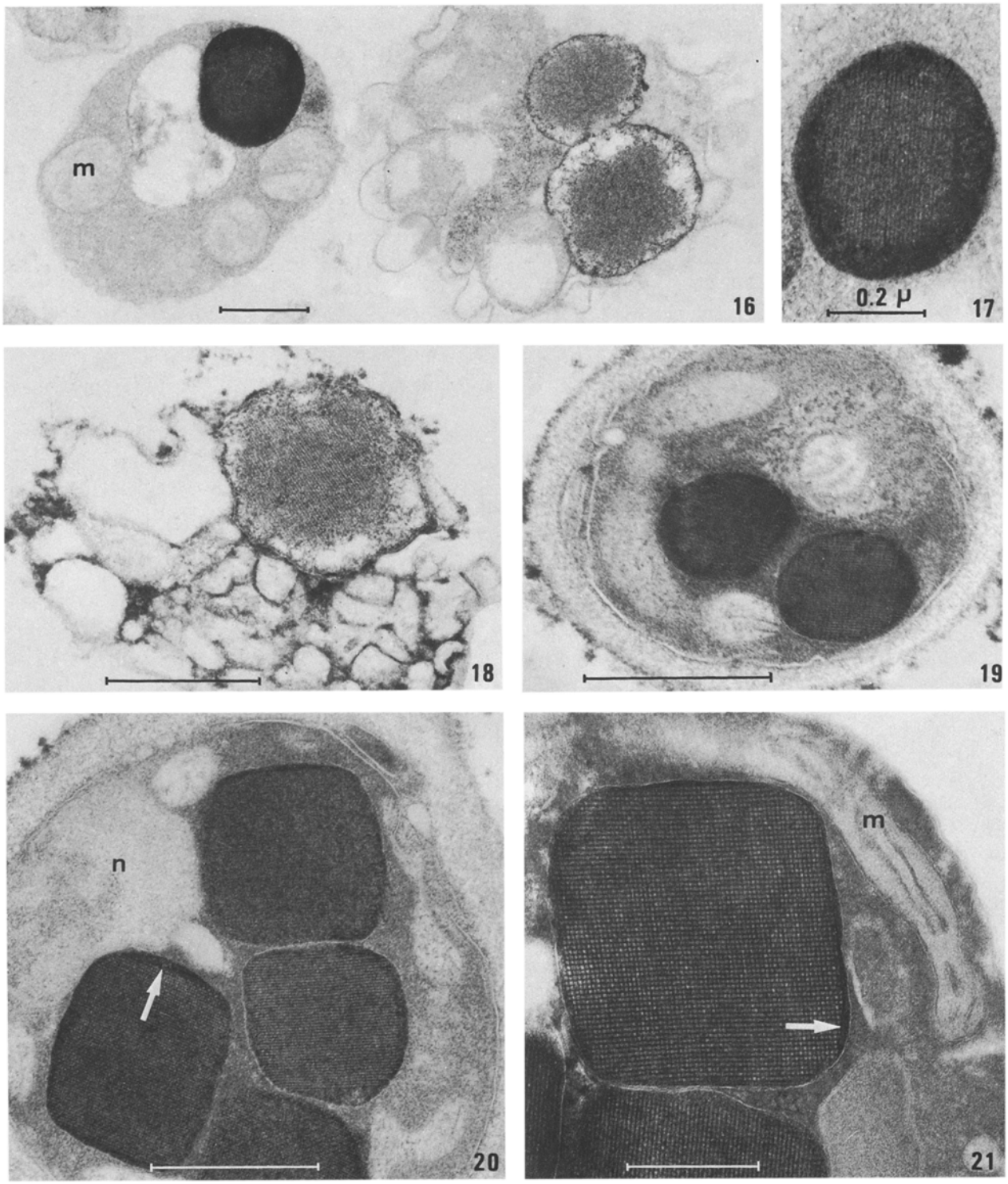


Fig. 16. Survey of an intact and swollen spheroplast after incubation with $CeCl_3$ and methanol. The non-crystalline part of the peroxisome in the intact spheroplast stained more intensive than the crystalloid. In the swollen spheroplast activity of alcohol oxidase is absent in the non-crystalline matrix of the peroxisome. Activity is still apparent in the crystalloid

Fig. 17. High magnification of a peroxisome, after incubation with $CeCl_3$ and methanol, to demonstrate the pattern of stain deposit

Fig. 18. Detail of a swollen speroplast after incubation with $CeCl_3$ and DL-alanine to demonstrate D-amino acid oxidase activity

Fig. 19. Intact spheroplast showing urate oxidase activity in the peroxisomes after incubation with $CeCl_3$ and urate

Figs. 20 and 21. The similarity in structure and pattern of stain deposit for different oxidase activities in peroxisomes of cells from late stationary batch cultures (Fig. 20) and chemostat cultures (Fig. 21; dilution rate 0.1 h^{-1}). Although they differ in size, the peroxisomes in both cells are cubic of form with a crystalline matrix. The small non-crystalline zone shows more intensive staining than the crystalloids (*arrows*). Figure 20 illustrates staining with $CeCl_3$ and D-alanine, Figure 21 staining with $CeCl_3$ and glycollate

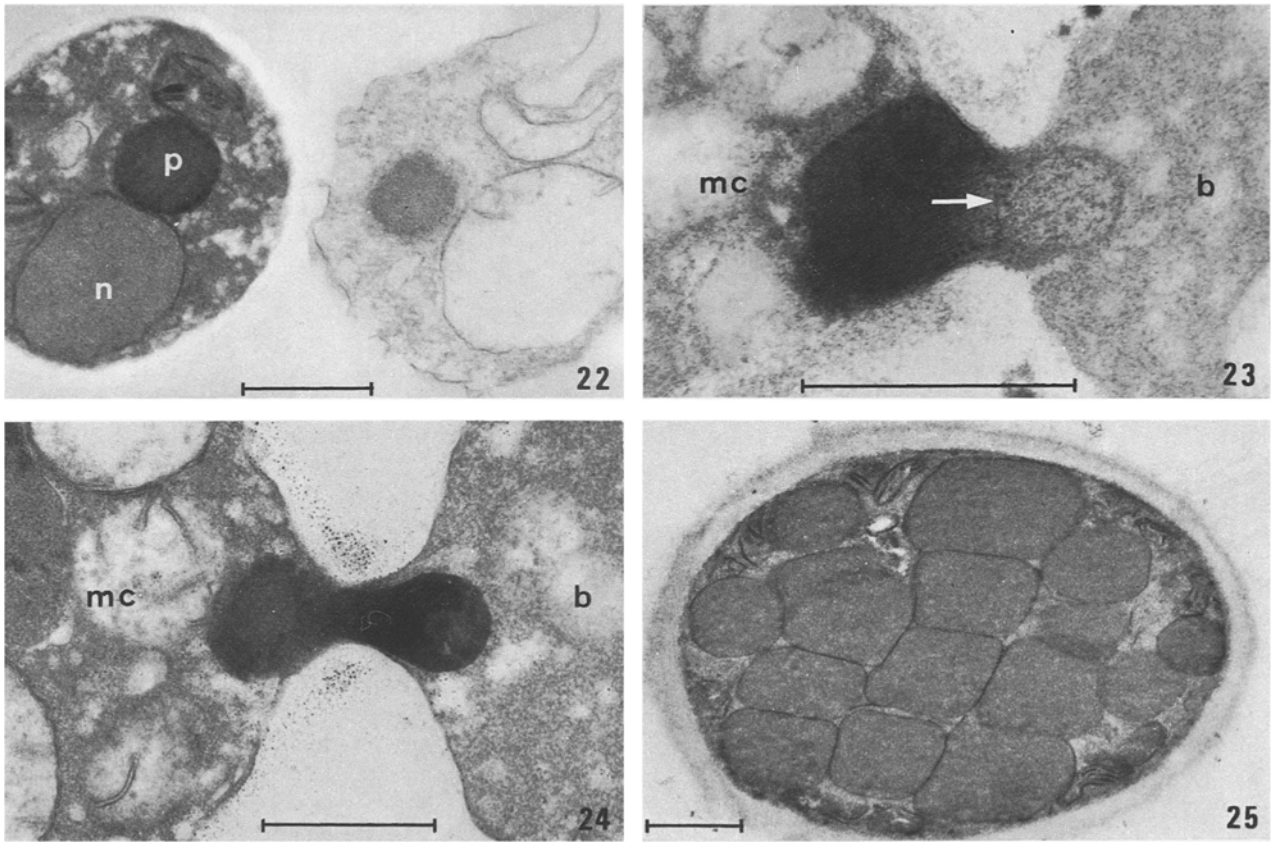


Fig. 22. Survey of an intact and swollen spheroplast after incubation with diaminobenzidine and H_2O_2. The soluble part of the intact peroxisome stained more intensive than the central crystalline part. The peroxisome in the swollen spheroplast hardly shows catalase activity

Fig. 23. Detail of a budding cell, incubated with $CeCl_3$ and methanol. Alcohol oxidase is hardly active in the separating peroxisome which migrates into the bud. Note the separating membrane (*arrow*)

Fig. 24. Detail demonstrating catalase activity in a dividing peroxisome in the neck between mother cell and bud after incubation with DAB and H_2O_2. In this figure the peroxisomal fraction in the bud stained more intensive due to absence of the crystalloid

Fig. 25. Section of an old mother cell from a chemostat culture (dilution rate $0.17\,h^{-1}$) showing numerous peroxisomal profiles

individual organelles. This increase in size was associated with an overall change in peroxisome morphology; mature peroxisomes in cells growing at a rate approaching μ_{max} (dilution rate $0.17\,h^{-1}$) were more or less round, whereas mature peroxisomes of cells growing at a dilution rate of $0.03\,h^{-1}$ were cubic of shape. However, independent of these morphological differences, the peroxisomes showed a completely crystalline matrix. Although in these studies it was impossible to perform a quantitative analysis of peroxisomal distribution in mother cells and daughter cells separately, the apparent difference in size between older cells and buds (Vraná et al., 1973; Vraná, 1974), especially at low growth rates, made a qualitative study of the development of peroxisomes in chemostat-grown cells possible. It was observed that old cells, characterized by the presence of many bud scars (Streiblova and Beran, 1963) contained large amounts of peroxisomes. Up to 18 peroxisomal profiles have been observed in such cells in which the volume of the peroxisomes contributed to 80% of that of the cytoplasm (Fig. 25). Developing buds contained generally 5–7 small peroxisomes, always of round shape with a completely crystalline matrix. These peroxisomes arose from mature peroxisomes in the mother cell by a mechanism identical to that described for cells grown in batch culture (Figs. 3–5). After separation from the mother cell, the peroxisomes in the bud increased in size, and finally became large mature peroxisomes. Multiplication of peroxisomes in these cells was brought about by the separation of generally one or two small peroxisomes from a mature one. They remained attached to the mature peroxisome and had a round shape with a completely crystalline substructure.

Discussion

Our experiments have shown that the growth conditions are of decisive importance for both the quantitative and qualitative aspects of peroxisome development during growth of *Hansenula polymorpha* on methanol.

During growth in batch culture the volume density of peroxisomes in the cells strongly increased (Fig. 1 D). The morphological data clearly demonstrated that this increase in volume density is not only caused by a gradual increase in number, but more in particular by a considerable increase in size of the individual peroxisomes. Growth of peroxisomes was found to be associated with an increase in the size of the crystalline nucleus in these organelles. Since the specific activity of (peroxisomal) alcohol oxidase and catalase of the culture also showed an enormous increase (Fig. 1 B) it is tempting to suggest that growth of the peroxisomes – and their crystalline inclusions – is related to increased amounts of the enzymes in the cells. However, catalase may not be involved in the formation of the crystalloids. This may be concluded from the experiments on osmotically shocked peroxisomes, since these organelles generally showed no catalase activity after incubation with DAB and H_2O_2. Incubations with DAB and methanol also failed to demonstrate any reaction product. Since incubations with $CeCl_3$ and methanol gave positive results, it was postulated that catalase had completely leaked from the swollen peroxisomes. Therefore, catalase is probably not integrated in the crystalloids since this structure, although catalase-negative, remained apparently intact. The correlation between the formation of crystalline inclusions in peroxisomes of methanol-grown *Candida boidinii* and the synthesis of alcohol oxidase has been shown in an elegant way by Sahm et al. (1975); peroxisomes in mutants of this organism which lacked alcohol oxidase activity did not contain crystalline inclusions, although catalase activity was present to the same extent as in wild type cells (Eggelink et al., 1977).

Besides alcohol oxidase other oxidases are most likely incorporated in the crystalloids, since cytochemical staining experiments showed activity of these enzymes in the crystalloids of osmotically shocked peroxisomes. However, when, similar to the situation in *Candida boidinii*, alcohol oxidase and catalase are the major components of peroxisomes (Roggenkamp et al., 1975) it is reasonable to assume that alcohol oxidase is quantitatively the most important component of the crystalloid. If this is generally true for peroxisomes of methanol utilizing yeasts, it follows that growth of the crystal during the development of *Hansenula polymorpha* in batch cultures is primarily due to an increase in the amount of alcohol oxidase in the cells. It may be speculated that environmental conditions such as decreasing methanol and oxygen concentrations in these cultures prescribe a higher level of alcohol oxidase in the cell as growth proceeds (van Dijken et al., 1976), which, when a critical concentration of the enzyme is reached in the organelles, results in the formation of a crystalline peroxisomal matrix. The observation that some cells in methanol-limited chemostat cultures growing at a low dilution rate may contain up to 18 peroxisomes, which make up 80 % of the volume of the cytoplasm, indicates that alcohol oxidase can be piled up to incredible levels in the cell. The fact that these cells were old mother cells indicates that such an accumulation of alcohol oxidase may also depend upon the time during which individual cells have been subject to growth-limiting conditions which affect the synthesis of the enzyme. It may well be that the only way in which the cells can handle such high levels of this peroxisomal enzyme is to deposit it in a crystalline structure. Cytochemical staining of alcohol oxidase always showed that the enzyme is less active in the crystalloid than in the non-crystalline matrix. A relative increase in the amount of crystalline enzyme as compared to soluble enzyme would therefore imply a decrease in the specific activity of alcohol oxidase in vivo. This was observed during growth of the yeast in batch cultures during which the peroxisomes became more and more crystalline; the capacity of whole cells to oxidize excess methanol, which is thought to reflect the in vivo alcohol oxidase activity (van Dijken et al., 1976), increased by a factor of 3, whereas the in vitro activity of alcohol oxidase increased by more than a factor of 10 (Fig. 1).

The increased rate of synthesis of alcohol oxidase in *Hansenula polymorpha* in the later stages of the batch cultures also affected the process of bud formation. In the early log phase, when the alcohol oxidase content and the number of peroxisomes in young cells was low, only 1 – 2 peroxisomes migrated to the buds. At the end of logarithmic growth, however, when the amount of enzyme and organelles reached their maximum, generally 2 – 4 peroxisomes were transferred to the bud. In cells of chemostat cultures, in which the amount of peroxisomes is higher than in cells of the decelerated growth phase (compare Fig. 1 C and Table 2), even 5 – 7 peroxisomes were donated to the bud. Among the factors which may control the process of peroxisomal multiplication, the rate of enzyme and organelle synthesis is probably not decisive. In chemostat cultures growing at a dilution rate of $0.03\,h^{-1}$ the level of alcohol oxidase was approximately twice that of cells growing in the late exponential phase in batch culture when the growth rate was $0.20\,h^{-1}$. The rate of enzyme synthesis in batch cultures of the late exponential phase is therefore approximately three times higher than in cells of this chemostat culture in which more organelles

were donated to the bud. It seems therefore likely that both the extent and the duration of environmental pressures to which the cells are exposed are involved in controlling the process of multiplication of the peroxisomes. Further studies on factors determining this apparently well-regulated process are in progress.

Migration of peroxisomes from mother cell into bud in batch cultures was found to be associated with a (temporary) loss of alcohol oxidase activity (Fig. 18). This observation is in agreement with those made on cells of chemostat cultures (Veenhuis et al., 1976). Catalase, however, remained active during this process. The process of inactivation of alcohol oxidase may be akin to that of its activation. The enzyme is synthesized on ribosomes, which are not present in the peroxisome itself. Cytochemical staining experiments on whole cells or spheroplasts never showed any cytoplasmic alcohol oxidase activity. A problem yet to be solved is the process of assemblage of alcohol oxidase and its transport into the peroxisomes where it becomes active. It is possible that inactive subunits and/or apoenzyme are intermediates in the biosynthesis of active alcohol oxidase, a process which may be similar to that of the biosynthesis of peroxisomal catalase as outlined by Lazarow and De Duve (1973). Activation or inactivation of alcohol oxidase may then be accomplished by association or dissociation of FAD which is bound non-covalently to each of the eight subunits of the enzyme (Sahm and Wagner, 1973; Kato et al., 1976). Whether such a process indeed occurs during peroxisome migration or during inactivation of the enzyme in the lag phase (Fig. 1 B) remains to be elucidated.

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